

# NREL-Amoco CRADA Phase 3

## Bench Scale Report 1.1

### Continuous Fermentation of Pure Sugars by LNH33C

**Project Title:** Amoco-NREL CRADA with corn fiber

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#### **Objective:**

To examine the capability of LNH33C to simultaneously ferment xylose and glucose at levels representative of pretreated corn fiber hydrolyzate and to demonstrate its ability to grow in continuous culture under conditions of interest to the Amoco-NREL CRADA.

#### **Background:**

The compositional make-up of pretreated corn fiber hydrolyzate prepared by Amoco was ascertained to determine the monomeric sugar loading of glucose and xylose. The levels of glucose and xylose were determined to be 2.4% w/v and 3.4% w/v respectively. The pure sugar fermentation was planned at these levels of sugars to mimic the hydrolyzate composition, but without including any potential inhibitors present in hydrolyzate.

#### **Materials and Methods:**

##### *Inoculum Preparation:*

A frozen (-70°C) stock vial of LNH33C was grown in 1% w/v corn steep liquor (CSL), 1% w/v yeast extract, 2% w/v peptone, and 2% w/v xylose. The flask contained a total volume of 50 mL in a 250-mL baffled Erlenmeyer flask and was incubated at 30°C with an agitation of 150 rpm. After 28 hours of growth, 10% v/v was transferred to 2% w/v CSL, 1% w/v yeast extract, and 2% w/v xylose for inoculum growth. This flask contained a working volume of 100 mL in a 500-mL baffled Erlenmeyer flask and again was incubated at 30°C with an agitation of 150 rpm.

##### *Fermentation Conditions:*

The fermentation was started in batch mode with 2% w/v CSL, 1% w/v yeast extract, 2.5% w/v glucose, and 3.4% w/v xylose as the medium. A 10% v/v inoculum was transferred to the fermentor vessel and was allowed to grow for 24 hours in batch before being switched to continuous. The feed for continuous mode consisted of the same medium as the reactor, but was made up in a 15-L batch with xylose and glucose being filter-sterilized and added after the yeast extract and CSL solution was autoclaved. The pH was adjusted to 5 in both the fermentor and the feed with 3N sodium hydroxide.

For the fermentation, a New Brunswick BIOFLO III fermentor was employed. To minimize ethanol evaporation, the condenser was packed with 1-mm glass beads (to maximize the surface area) and equipped with 4°C water circulation. The working volume of the vessel was 1 L, agitation was controlled at 150 rpm, temperature was maintained at 30°C and, the pH was

maintained at 5 with either the addition of 3N sodium hydroxide or 1% w/v sulfuric acid. Air was not supplied to the fermentation.

#### *Analysis:*

Samples were withdrawn at regular intervals and analyzed on the Yellow Springs Instrument (YSI) for ethanol and glucose. In addition, samples were analyzed on a Hewlett Packard 1090 HPLC for glucose, xylose, succinic acid, acetic acid, lactic acid, glycerol, and ethanol. Optical density at 600 nm (OD) and dry cell weight were obtained to monitor cell growth on every sample. The viability of the cells in the fermentor was determined on a daily basis and the plasmid stability was also monitored.

#### **Results and Discussion:**

In batch mode the glucose was rapidly consumed. The xylose was not utilized until the glucose was almost completely consumed (see figure 1). The fermentation was switched to continuous mode after 24 hours in batch. At this point all of the glucose had been consumed, while 20 g/L xylose still remained. At a residence time of 74 hours it took sometime to reach a steady state, at which point the glucose remained null, whereas the xylose reached a minimum of 5 g/L and increased to 25 g/L until it reached a steady state. Glycerol was the main by-product produced (3.5 g/L at steady state) with no discernible succinic acid, lactic acid, or acetic acid production. The production of xylitol mirrored the production of glycerol. The OD was ~ 3 and the dry cell weight was ~2.25 g/L at the 74 hour residence time steady state (see figure 2).

It appears that the xylose was not co-metabolized with glucose, as the xylose was not consumed until the glucose was nearly depleted. It is interesting to note that the xylose concentration reached a minimum level of 5 g/L, but was not maintained at this level at a 74 hour residence time. In batch mode and shortly after being switched to continuous mode, the cell mass increased to a high of 6.5 g/L. With this high cell mass, xylose was nearly depleted. However with the decline in the cell mass as the fermentation strived to reach a steady state, the xylose concentration increased. This indicates that a cell mass of greater than 6 g/L will be required to utilize all of the xylose with a residence time of 74 hours. Therefore, in order to deplete the xylose at a residence time of 74 hours, continuous inoculum may have to be supplied to the fermentation. Further experimentation needs to be carried out to determine if continuous inoculum will help deplete the available xylose.

**Table 1:** Sugar concentration profile during the batch phase.

Time (h)	Glucose (g/L)	Xylose (g/L)	Xylitol (g/L)
0	25.51	38.44	0.13
3	21.18	38.62	0.08
6	4.43	37.77	0.10
21.5	0	19.14	2.12
24	0	15.8	2.47

After switching to continuous mode, the cell mass increased to a high of 6.5 g/L. At this cell mass, xylose was nearly depleted with only 6 g/L remaining in the fermentor. As the fermentation attained steady state, the cell mass declined to 2.25 g/L and the xylose concentration increased to 25 g/L (Figure 1). This indicates that a cell mass concentration greater than 2.25 g/L may be required to utilize all of the xylose at a residence time of 74 hours in a single stage. One way to achieve significantly higher steady-state cell mass concentration is through continuous inoculation.

Glycerol was the main by-product (3.5 g/L at steady state). No discernible amounts of succinic or acetic acid were produced, and lactic acid formation was rather negligible. The production of xylitol mirrored the production of glycerol. The OD was ~ 3 and the dry cell weight was ~2.25 g/L at the 74 hour residence time steady state (Figure 2).

Yield calculations and sugar conversions were based on the average values of ethanol, glucose, and xylose from the 74-h steady state time points 310 hours through 432 hours (Figure 1). The overall C6-sugar conversion was nearly complete at 99.5%. The overall C5-sugar conversion was 44.5%. The ethanol metabolic yield was 77.7% of theoretical, whereas the process yield was only 50.4% of theoretical (Table 2). *Process yield* is based on available fermentable sugars, whereas *metabolic yield* is based on actually consumed sugars. In addition to the sugar conversions and ethanol yield calculations, the distribution of products was determined for the 74-h steady state (Figure 3). The overall carbon balance closure was satisfactory (94.9%).

**Table 2:** Fermentation performance at steady state (74-h residence time).

Overall C6-sugar conversion	99.5%
Overall C5-sugar conversion	44.5%
Ethanol process yield (% theoretical)	50.4%
Ethanol metabolic yield (% theoretical)	77.7%
Ethanol volumetric productivity (g/L h)	0.244
Ethanol specific productivity (h <sup>-1</sup> )	0.112

After the 74-h residence time steady state had been sustained for two residence times, the residence time was decreased to 60 hours (Figure 1). Decreasing the residence time did not appear to have an effect on the concentrations of glucose, glycerol, xylitol, cell mass, and ethanol. A decrease observed in the xylose level after the residence time was lowered to 60 hours was due to an unexpected change of the xylose concentration in the feed vessel from the original concentration of 45.78 g/L down to 29.68 g/L (in contrast, the glucose concentration decreased only slightly from 26 g/L to 24 g/L). The disappearance of the xylose in the feed cannot be attributed to contamination, since microscopic evaluation and plating did not detect such an event. A plausible explanation is that xylose may be polymerizing after long exposure to room temperature.

Subsequently, the residence time was decreased to 44 hours in an attempt to determine the effect of shorter residence time on the fermentation. At the same time, new feed was prepared and connected to the fermentor. Again, very little effect on glycerol, xylitol, glucose and cell mass was observed. Due to the introduction of a fresh feed (as explained in the previous paragraph), the xylose concentration returned to approximately its original steady state level. The fermentation was intentionally terminated at 864 hours.

It is interesting to note that there was very little, if any, effect on the fermentation performance by decreasing the residence time from 74 to 44 hours. At all residence times all of the glucose was consumed and the same amounts of ethanol, cell mass, and by-products were generated (unfortunately, the problem with the xylose feed does not allow any conclusions to be drawn regarding xylose utilization at steady states other than the 74-h setting). This outcome is not surprising given the fast doubling time of the microorganism during glucose (2.1 hours) and xylose (5.3 hours) fermentation, as described in Report 1.3. In other words, even at a 44-h residence time, the cells have sufficient time to consume all the glucose (and some xylose) provided and produce similar amounts of ethanol.

The viability and plasmid stability were followed throughout the fermentation. The viability of the culture decreased throughout the run from around 60-70% to 30-40%. However, it should be pointed out that there are numerous inherent shortcomings with this routinely used plating viability method. In contrast, the more reliable epi-fluorescence assay (developed at NREL) yielded a viability figure of 93%, when the plating method reading was only 56%. At the present time, the lack of fluorescence capability in our microscope does not allow us to use the epi-fluorescence method on a routine basis. With respect to plasmid stability, it decreased slightly to 98% at the end of the study. Unfortunately, the plasmid stability method does not give us an understanding of the plasmid copy number, but only indicates if the plasmid is present.

### **Conclusions and Recommendations**

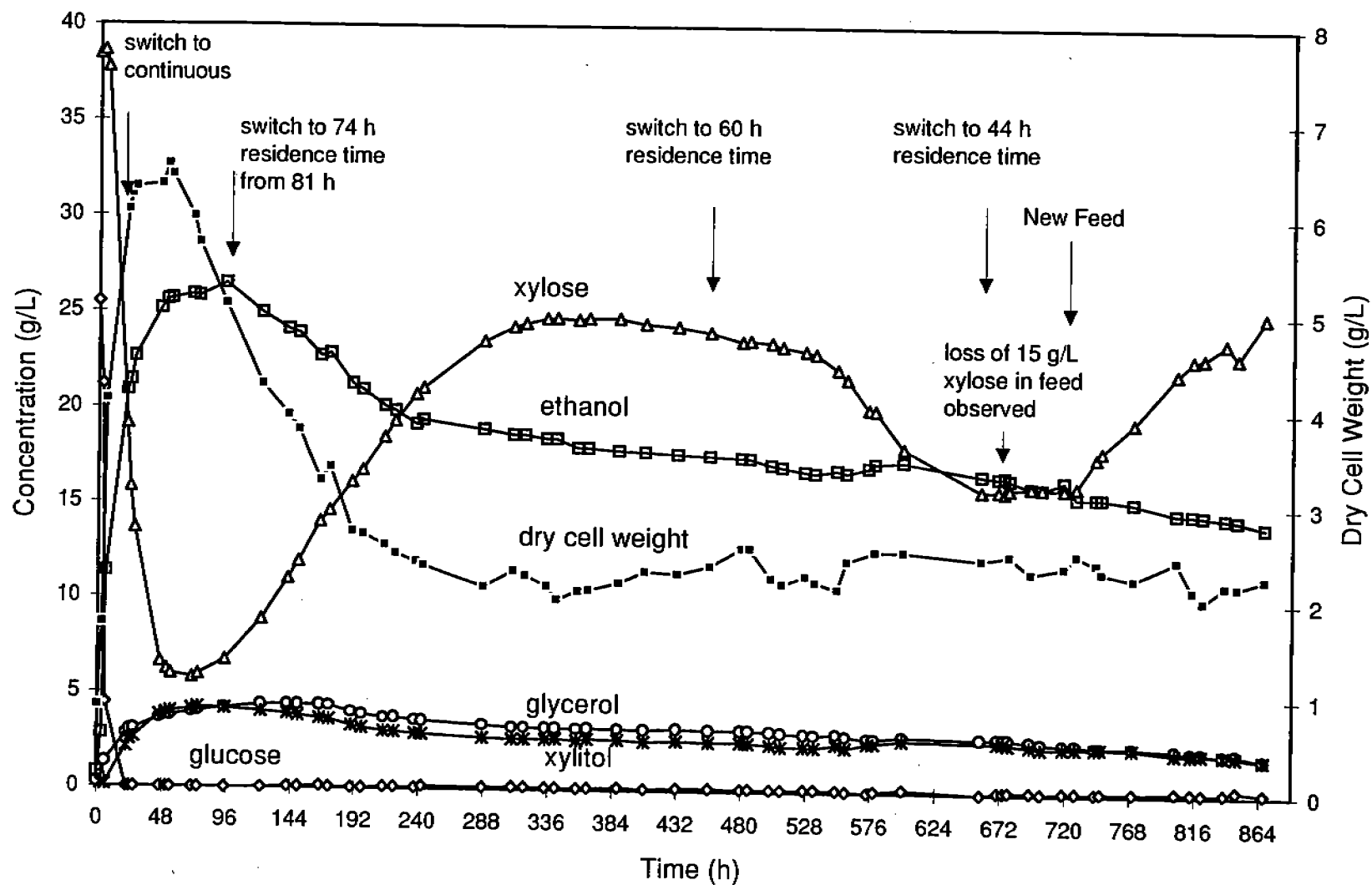
In continuous mode, all of the glucose was consumed regardless of the residence time. Xylose was only partially utilized: about 19 g/L xylose (44 %). Decreasing the residence time from 74 to 44 hours seemed to have little effect on the cell mass, glycerol, xylitol, and ethanol concentrations.

Because a single-stage system cannot mimic the 3-stage configuration of the PDU, it would be of interest to test this organism or any of its descendants in a multi-stage fermentation system in the laboratory. This would allow the glucose to be consumed in the first stage, and then subsequent stages would only see a minimal amount of glucose, hence "forcing" the organism to utilize xylose.

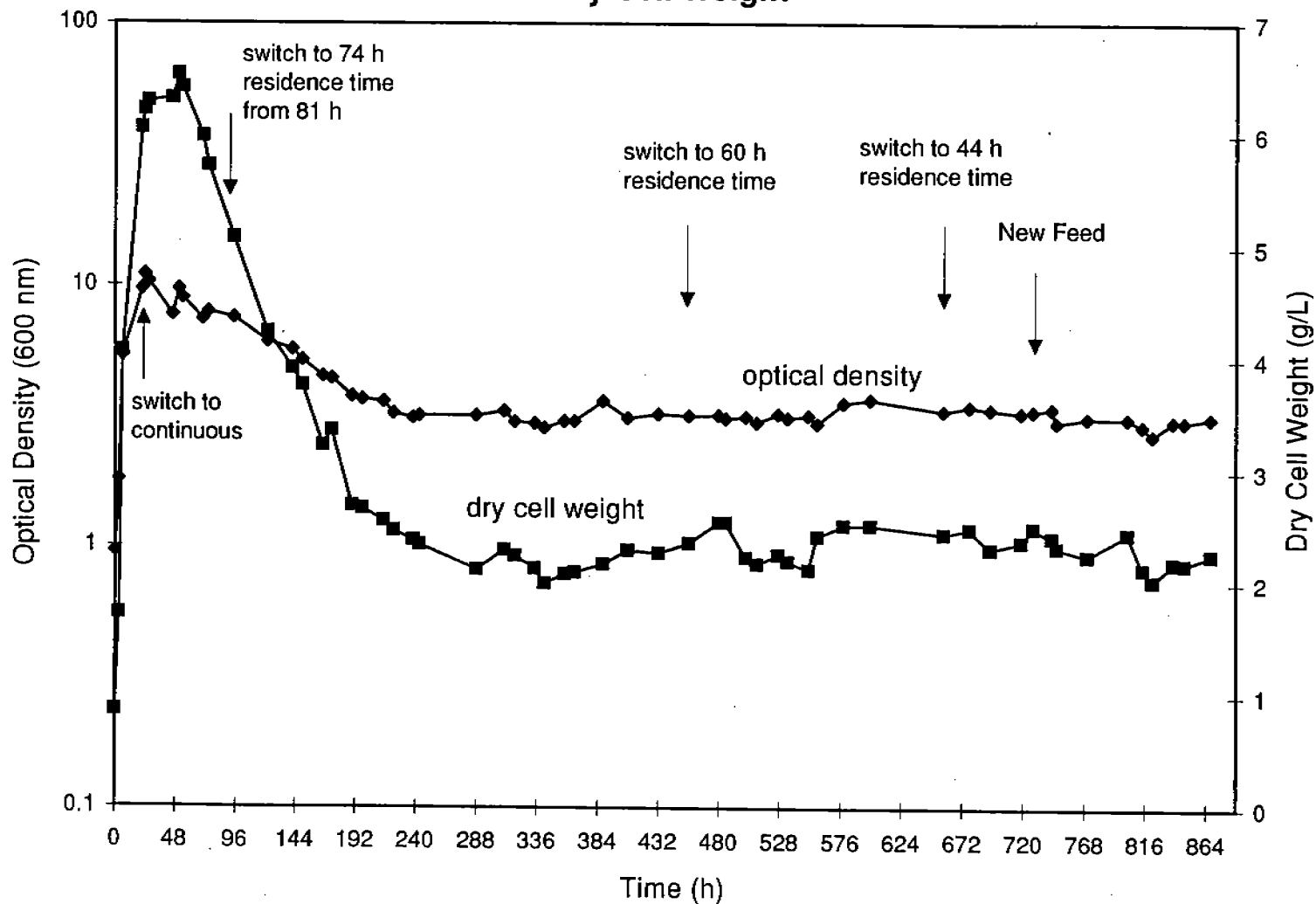
The organism does, however, show better potential in producing ethanol than its parent strain, L1400, since it can also convert xylose to ethanol.

The loss of xylose in the feed was troubling, but with this information in hand we should be able to avoid similar situations in the future by using smaller feed batches for long experiments and keeping a close eye on the feed composition.

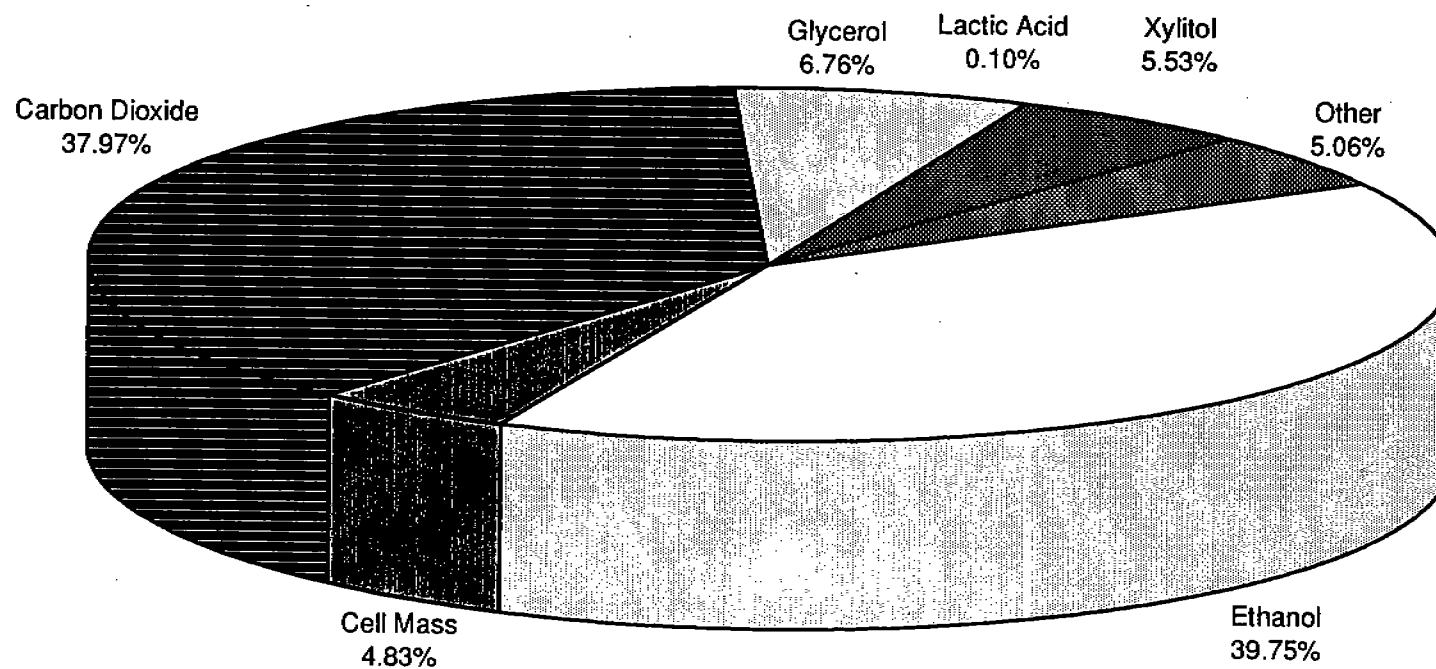
Figure 1: Pure Sugar Continuous Fermentation by LNH33C



**Figure 2: Growth of LNH33C as Determined by Optical Density and Dry Cell Weight**



**Figure 3: Product Distribution per 100 g of C5s and C6s Converted**



**Carbon Balance Closure: 94.9%**